Virology 270, 65–75 (2000)

Homologous Recombination between a Lactococcal Bacteriophage and the Chromosome of Its Host Strain

Julie D. Bouchard and Sylvain Moineau[1]

Department of Biochemistry and Microbiology, Faculté des Sciences et de Génie, and Groupe de Recherche en Écologie Buccale (GREB), Faculté de Médecine Dentaire, Université Laval, Québec, G1K 7P4 Canada

Received December 13, 1999; returned to author for revision January 10, 2000; accepted January 28, 2000

Genetic exchanges constitute a significant means by which bacteriophages acquire novel characteristics. Phages of *Lactococcus lactis* occupy a particular niche, the dairy factory environment, where their populations are subjected to constant changes. Little is known about the mechanisms of evolution that lead to the genetic diversity of lactococcal phages. In this study, we described two DNA exchanges involving the lytic phage ul36, a member of the P335 species, and its *L. lactis* host. They occurred by homologous recombination with phage-related sequences present in the host chromosome. Both mutants generated by these recombination events are insensitive to the phage resistance mechanism AbiK and one has a reduced burst size as well as a new origin of replication. We propose that this type of DNA exchange with prophages or remnants of prophages occurs frequently within the P335 species as supported by DNA-DNA comparisons between P335-like phages.

INTRODUCTION

It has long been known that bacteriophages belonging to the same DNA homology group can exchange genetic material (Casjens *et al.,* 1992). Even remnants of prophages can contribute to the phage gene pool by recombining with invading phages (Campbell, 1988). New genomic combinations can be created by at least four different mechanisms: homologous, microhomologous, site-specific, and illegitimate recombinations (Casjens *et al.,* 1992). Homologous recombination, which relies on the presence of homologous DNA sequences and site-specific recombination, which is also used by temperate phages to integrate into the host chromosome, are believed to be the most frequent modes of DNA exchange (Campbell, 1994).

Lactococcus lactis is a gram-positive lactic acid bacterium added to milk to drive the fermentation during the manufacture of products such as cheeses, sour cream, and buttermilk. Lactococcal lytic bacteriophages are the leading agents of fermentation failures in the dairy industry worldwide. The dairy plant environment is a particular niche with regard to the phage propagation, because there is a very high concentration of bacteria and relatively few host strains. Phage control strategies are now relying on the use of genetically modified *L. lactis* strains containing natural antiphage mechanisms (Moineau, 1999). To multiply in that setting, therefore, a phage must avoid or circumvent the host's defense systems. These circumstances have led to the emergence of phage mutants that are insensitive to the antiphage barriers (Alatossava and Klaenhammer, 1991). With the increasing number of distinct phage-resistance mechanisms available to the industry, we can expect a diversification of the phage population and even the appearance of "multiresistant" phages, as a result of these various selective pressures. A general knowledge of phage evolution is essential for the design of long-term phage-resistant strains. For these reasons, a better understanding of the mechanisms of evolution intervening in the different lactococcal phage species is necessary.

Lactococcal bacteriophages have been divided into twelve species, essentially according to DNA homologies (Jarvis *et al.,* 1991). Only three of these genetically distinct species are frequently encountered in the North American dairy factories: 936, c2, and P335 (Moineau *et al.,* 1992, 1996; Bissonnette *et al.,* 2000). All of the 936- and c2-like phages described to date are lytic, whereas the P335 species contains lytic and temperate phages. Lysogeny seems frequent among *Lactococcus* strains and the great majority of prophage-related sequences analyzed thus far showed homology to P335-like phages (Djordjevic and Klaenhammer, 1997; Prévots *et al.,* 1998; Labrie and Moineau, 2000). Consequently, lytic phages of this species could potentially exchange genetic material with chromosomal DNA and acquire new distinctive features.

Two DNA exchange events involving P335-like phages have already been reported (Hill *et al.,* 1991b; Moineau *et al.,* 1994). The recombinant phages were mutants, resistant to a natural antiphage system. The source of the

[1] To whom correspondence and reprint requests should be addressed at Université Laval, Groupe de Recherche en Écologie Buccale (GREB), Faculté de Médecine Dentaire, Québec, G1K 7P4 Canada. Fax: 418-656-2861. E-mail: Sylvain.Moineau@bcm.ulaval.ca.

0042-6822/00 $35.00

TABLE 1

Bacterial Strains, Bacteriophages and Plasmids Used in this Study

Bacterial strains, phages, or plasmids	Relevant characteristics[a]	Source
L. lactis		
SMQ-86	Multiple plasmids, host for P335 phages, AbiK$^-$, Em^r	Emond *et al.,* 1997
SMQ-88	Multiple plasmids, host for P335 phages, AbiK$^+$, Em^r	Emond *et al.,* 1997
SMQ-481	NCK203 partially cured of wild-type plasmids, host for P335 phages	This study
SMQ-482	SMQ-481(pMIG3), Cm^r	This study
SMQ-483	SMQ-481(pSRQ828), Cm^r	This study
SMQ-484	SMQ-481(pSRQ829), Cm^r	This study
E. coli		
DH5α	Cloning host	Gibco/BRL
SMQ-396	DH5α(pSRQ825), Ap^r	This study
SMQ-397	DH5α(pSRQ826), Ap^r	This study
SMQ-398	DH5α(pSRQ827), Ap^r	This study
Bacteriophages		
ul36	P335 species, small-isometric head, AbiK^s	Moineau *et al.,* 1992
ul36.1	P335 species, small-isometric head, mutant of ul36, AbiK^r	This study
ul36.2	P335 species, small-isometric head, mutant of ul36, AbiK^r	This study
ϕ31	P335 species, small-isometric head	Alatossava and Klaenhammer, 1991
ϕ50	P335 species, small-isometric head	Alatossava and Klaenhammer, 1991
Q30	P335 species, small-isometric head	Moineau *et al.,* 1996
Q33	P335 species, small-isometric head	Moineau *et al.,* 1996
Plasmids		
pBS	Cloning vector for sequencing, Ap^r, 2.9 kb	Stratagene
pMIG3	Shuttle vector, Cm^r, 5.5 kb	Wells *et al.,* 1993
pSRQ825	2.2-kb *Bgl*II-*Eco*RV fragment of ul36 cloned into pBS	This study
pSRQ826	4.0-kb *Bgl*II-*Apa*I fragment of ul36.1 cloned into pBS	This study
pSRQ827	1.7-kb *Bgl*II-*Eco*RV fragment of ul36.2 cloned into pBS	This study
pSRQ828	1.1-kb *Sca*I-*Dra*I fragment of ul36 cloned into pMIG3	This study
pSRQ829	1.1-kb *Apa*I-*Pst*I fragment of ul36.1 cloned into pMIG3	This study

[a] AbiK^s, sensitive to AbiK mechanism; AbiK^r, insensitive to AbiK mechanism; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance, Em^r, erythromycin resistance.

acquired DNA was a plasmid (Hill *et al.,* 1991b) or the host chromosome (Moineau *et al.,* 1994). The mode of recombination was investigated in one instance where homologous recombination was excluded. Recently, we reported a high genomic diversity among several members of the P335 species (Labrie and Moineau, 2000). Comparative analysis between complete or partial genomic sequences may provide precise information regarding the evolutive history of this species.

In this study, we describe two distinct genetic exchanges that occurred through homologous recombination between a P335 phage and the chromosome of its host strain. The putative genes involved are located in the early region of the phage genome. The two recombinant phage mutants are insensitive to a natural antiphage mechanism (AbiK) and one of them has acquired a new origin of replication.

RESULTS

Phage isolation and microbiological characterization

L. lactis SMQ-88, a strain containing the abortive infection mechanism AbiK, was challenged with a high titer (10^{10} pfu/mL) of ul36, a lytic phage sensitive to AbiK (Table 1). Two phage mutants, named ul36.1 and ul36.2, were repeatedly isolated at a frequency of 10^{-6}. The mutants had an efficiency of plaquing (EOP) and an efficiency to form centers of infection (ECOI) of 1.0 on SMQ-88. Phage ul36.1 formed smaller plaques and had a significantly lower burst size than ul36 and ul36.2 on both SMQ-86 (AbiK$^-$) and SMQ-88 (Table 2). The presence of AbiK had no effect on the burst size of phages ul36.1 and ul36.2. Electron microscopy observations showed that both phages had the same morphology as ul36 (data not shown). Separation of the structural proteins by SDS–PAGE also revealed an identical profile for all three phages (data not shown).

TABLE 2

Burst size of Phages ul36, ul36.1, and ul36.2 in Absence or in Presence of AbiK[a]

Strain	ul36	ul36.1	ul36.2
SMQ-86	395 ± 33	284 ± 38	375 ± 39
SMQ-88	36 ± 27[b]	303 ± 30	420 ± 50

[a] $n = 3$.
[b] Emond *et al.,* 1997.

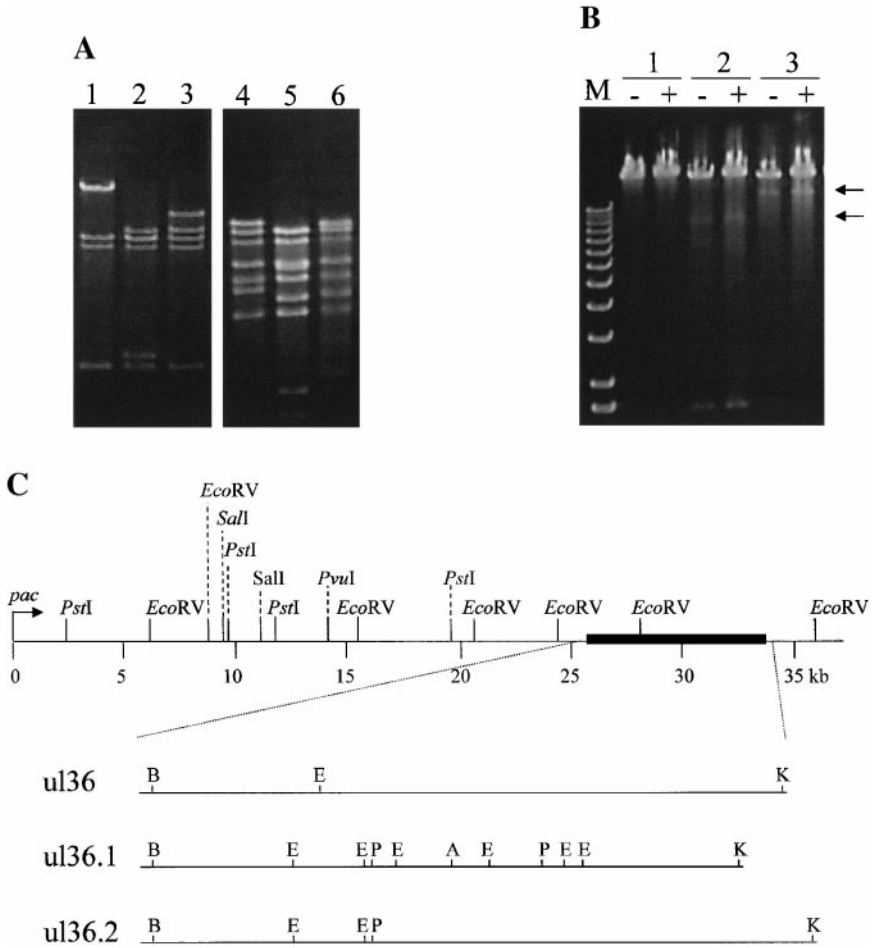

FIG. 1. Restriction profiles and maps of phages ul36, ul36.1, and ul36.2. (A) *Pst*I and *Eco*RV digestions. Lane 1, ul36; lane 2, ul36.1; lane 3, ul36.2 digested with *Pst*I. Lane 4, ul36; lane 5, ul36.1; lane 6, ul36.2 digested with *Eco*RV. (B) Determination of the *pac*-site location of ul36. Lane M, 1-kb ladder (1.6, 2.0, 3.0, 4.0, 5.1, 6.1, 7.1, 9.2, 10.2, 11.2, and 12.2 kb; Gibco/BRL, Burlington, Ontario, Canada); lane 1, undigested DNA of ul36; lane 2, *Sal*I digestion of ul36 DNA; lane 3, *Pvu*I digestion. −, without DNA ligase; +, DNA ligase treatment. (C) Restriction map of ul36 and comparison of the different regions with ul36.1 and ul36.2. B, *Bgl*II; E, *Eco*RV; P, *Pst*I; A, *Apa*I; K, *Kpn*I.

Restriction mapping of the phage genomes

The two mutants and the wild-type phage had distinct DNA restriction profiles (Fig. 1A). The restriction map of ul36 was established to localize the genomic differences. The map of approximately 37.3 kb was circular and heating at 75°C for 10 min or ligation of the DNA did not change the restriction profiles (Fig. 1B). These observations are typical of DNA packaging that occurs via a headful mechanism. Presence of bands in submolar concentrations allowed the localization of a putative *pac*-site (Fig. 1B). Although the genomic modifications found in ul36.1 and ul36.2 were different, they both mapped in the same region. A DNA fragment seemed to have been exchanged in both phages, since the total length of their genome was comparable to the genome of the wild-type phage ul36 (Fig. 1C).

Source of the exchanged DNA

The *Bgl*II-*Apa*I DNA fragment of ul36.1 and the *Bgl*II-*Eco*RV fragment of ul36.2 (Fig. 1C) were labeled and used to probe the chromosome of the host strain. Identical chromosomal restriction fragments hybridized with both probes (Figs. 2, B and C), except for a 2-kb *Pst*I fragment that hybridized only with the ul36.1 probe (Fig. 1C). The *Apa*I-*Kpn*I fragment of ul36.1 was also used as

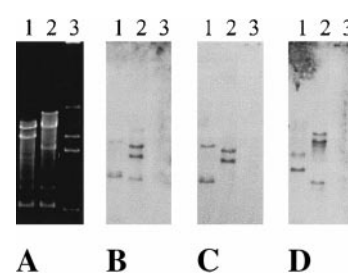

FIG. 2. Hybridization of the total DNA from *L. lactis* SMQ-86 with restriction fragments from the phages ul36.1 and ul36.2. Lane 1, total DNA of SMQ-86 digested with *Pst*I; lane 2, *Eco*RV; lane 3, undigested plasmid DNA of SMQ-86. (A) agarose gel; (B) autoradiogram following hybridization with *Bgl*II-*Apa*I fragment of ul36.1; (C) hybridization with *Bgl*II-*Eco*RV fragment of ul36.2; (D) hybridization with *Apa*I-*Kpn*I fragment of ul36.1.

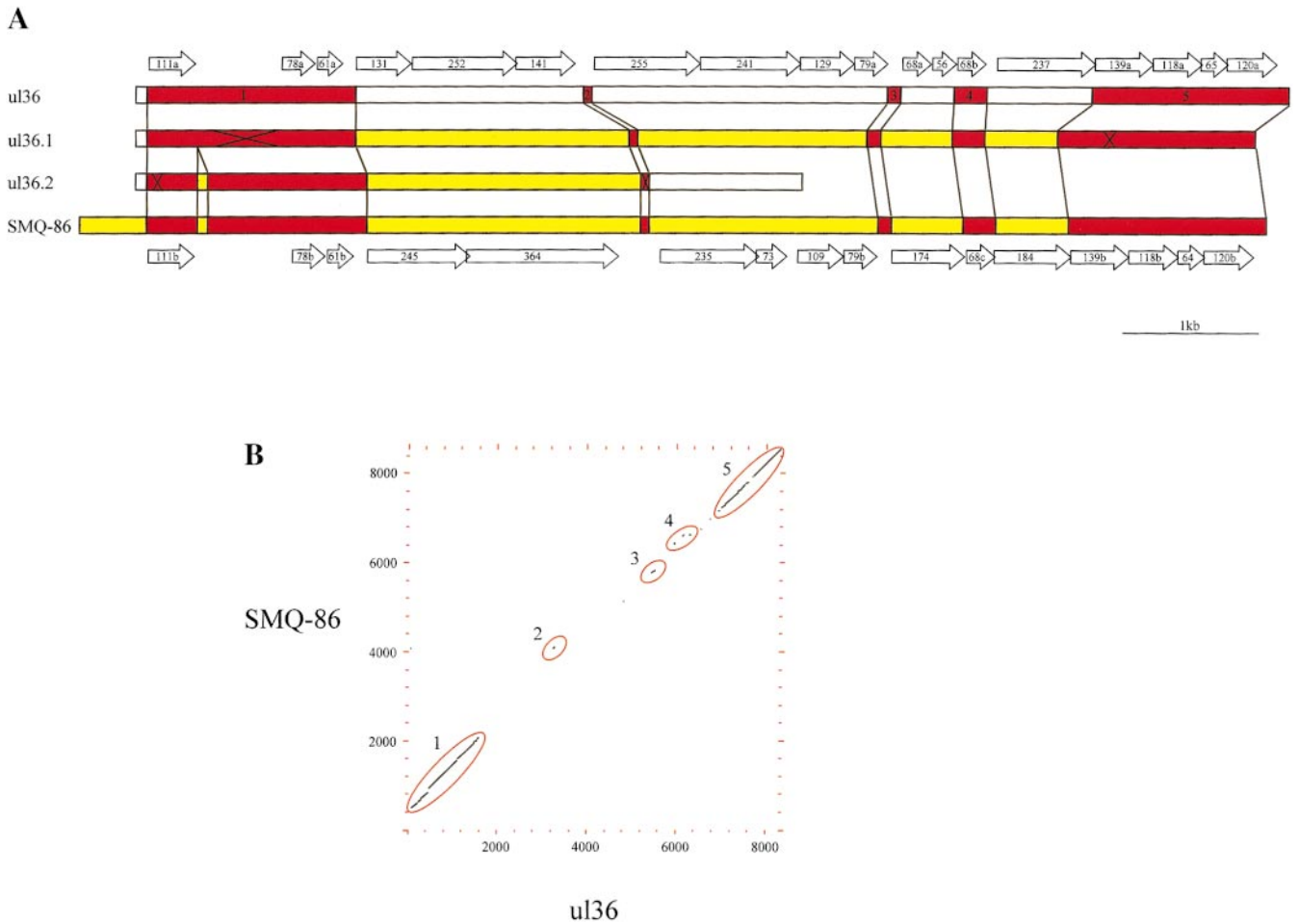

FIG. 3. (A) DNA homology between phages ul36, ul36.1, ul36.2, and the chromosome of *L. lactis* SMQ-86. In white, DNA of ul36; in yellow, chromosomal DNA; in red, regions of homology between ul36 and the chromosome. x, location of the junction points determined by the last divergent nucleotide between the phage mutant and ul36 or the chromosome. (B) DNA homology between ul36 and SMQ-86. The figure was created with the GCG compare and dot-plot programs (window of 21 and stringency of 19) and with GCG Figure. Regions of homology represented in (A) are circled in red.

a probe and hybridized with the 2-kb *Pst*I fragment and with other chromosomal fragments (Fig. 2D). No hybridization signal was observed with plasmid DNA of the host strain (Fig. 2). These results demonstrate that the DNA acquired by the phage mutants came from the chromosome. Furthermore, a common chromosomal region, in all likelihood, is present in both phages.

DNA sequencing and sequence analysis

The genomic regions of the three phages that presented differences were cloned in pBS vector and then sequenced by genome walking. The chromosomal DNA presumably acquired by ul36.1 and ul36.2 was sequenced with the use of PCR products obtained from *L. lactis* SMQ-86. Flanking regions of this chromosomal DNA obtained by inverse PCR were also sequenced. Comparison of the DNA sequences of ul36 and the host revealed five regions of high homology interspersed with large segments of significantly lower homology (41 to 47%) (Fig. 3). The characteristics of the homologous zones are shown in Table 3. The DNA sequences of ul36.1 and ul36.2 that were not homologous to ul36 were identical to the DNA of SMQ-86. The junction points of these units of genetic exchange were located within the regions of homology between ul36 and the chromosome (Fig. 3).

The G + C content was 35.4% over 8320 bp for ul36

TABLE 3

Characteristics of the Homologous Regions between Phage ul36 and the Chromosome of *L. lactis* SMQ-86

Region	Length (bp)	Percentage of homology (%)	Longest stretch of 100% homology (bp)
1	1515	96.8	468
2	55	92.7	29
3	91	93.4	37
4	231	67.4	24
5	1411	93.3	545

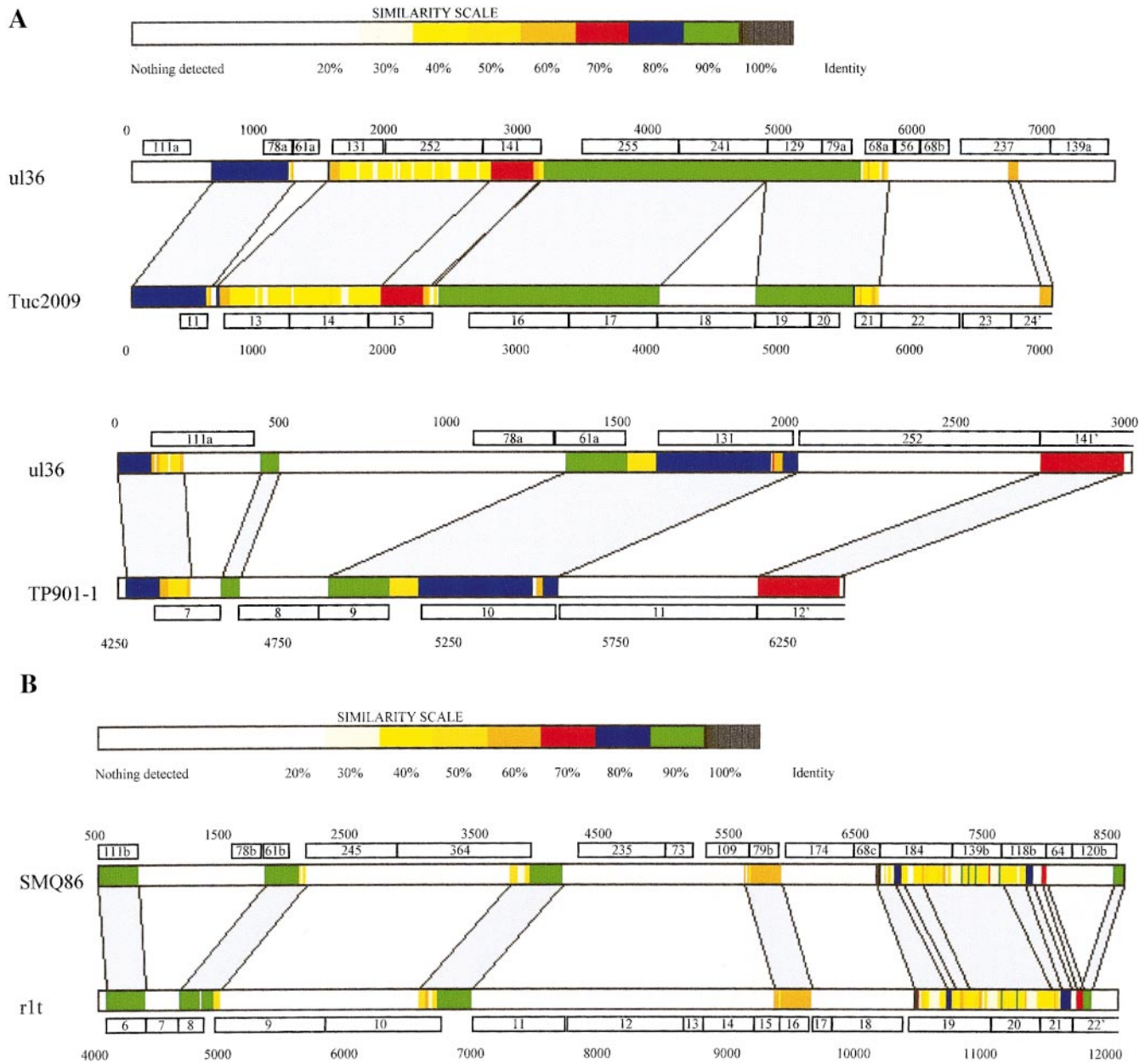

FIG. 4. Pairwise DNA sequence alignments of phage ul36 (A) and *L. lactis* SMQ-86 (B) with three members of the lactococcal phage P335 species. The predicted open reading frames (ORFs) are identified in boxes. The length of the box is proportional to the length of the predicted ORF. The figure was created with the SIM alignment tool and LaInview (Huang and Miller, 1991; http://www.expasy.ch).

and 34.1% over 8552 bp for SMQ-86. Open reading frames (ORFs) of more than 50 amino acids were identified and named according to the size of their putative products (Fig. 3, Tables 4 and 5). Several ORFs are overlapping and the longest noncoding sequence contains 707 bp and is located between ORF111b and ORF78b, in SMQ-86. This compact genetic organization is a general feature of phage genomes. Following the observation of similarities with the databases, putative functions have been assigned to ORF141 and ORF255 of ul36, which would encode a single-stranded binding protein and a replisome organizer, respectively. The putative gene products of ORF139a and ORF139b also have homology with dUTPases.

DNA-DNA comparisons were also performed with three temperate phages of the P335 species for which complementary information was available, namely Tuc2009, r1t, and TP901–1 (Fig. 4) (van Sinderen *et al.,* 1996; Madsen and Hammer, 1998; McGrath *et al.,* 1999). The patterns of DNA homology resemble the one observed with ul36 and SMQ-86. Regions of homology included one or two putative genes and were interspersed with areas of less than 50% homology comprising one or more putative genes. Interestingly, the puta-

TABLE 4

General Features of the Putative ORFs of the Sequenced Region of Phage ul36 and Comparison with Databases

ORF	Start	Stop	%G + C	MW (kDa)	pI	SD sequence AGAAAGGAGGA	Function or similarity	Percentage of identity (aa)	Accession no.
111a	92	427	33.0	12.9	5.21	AGAGAGGAAAAtcc	BK5-T (ORF111)	98 (109/111)	L44593
						ATG	rlt (ORF6)	97 (106/109)	U38906
78a	1059	1295	38.4	9.7	9.66	AGAAAGGAAAAta	phi31 (ORF71)	46 (34/73)	U51128
						ATG	Tuc2009 (ORF11)	46 (34/73)	AF109874.1
61a	1314	1499	33.9	7.1	5.19	Nonidentified	rlt (ORF8)	89 (52/58)	U38906
							TP901-1 (ORF9)	86 (50/58)	Y14232
131	1598	1993	36.1	14.9	6.59	AGAAACGGAGaacatt	TP901-1 (ORF10)	80 (106/131)	Y14232
						ATG			
252	2002	2760	36.9	28.8	5.24	GAGGtaaatc	Unknown		
						ATG			
141	2753	3178	36.2	15.7	5.41	AAAGGAAGAacaaa	SSB		
						ATG	Tuc2009 (ORF15)	69 (98/141)	AF109874.1
							TP901-1 (ORF12)	74 (57/77)	Y14232
							phiPVL (ORF45)	54 (77/141)	AB009866.1
							SPP1 (gp36)	40 (56/140)	X97918
255	3318	4085	33.5	28.7	5.70	AGAAAGGAGTAttc	Replisome organizer		
						gtggcacaaagaaga	Tuc2009 (Rep2009)	94 (241/255)	AF109874.1
						ATG	rlt (ORF11)	29 (44/150)	U38906
241	4085	4810	35.1	27.2	5.78	GGAGGtcttatcta	Tuc2009 (ORF17)	91 (221/241)	AF109874.1
						ATG			
129	4807	5196	34.9	15.7	9.73	ACAGGACAAcaaag	Tuc2009 (ORF19)	86 (112/129)	AF109874.1
						ATG	rlt (ORF14)	87 (112/128)	U38906
79a	5199	5438	29.2	9.5	9.64	Nonidentified	rlt (ORF16)	96 (76/79)	U38906
							Tuc2009 (ORF20)	89 (71/79)	AF109874.1
68a	5549	5755	32.9	8.2	9.69	Nonidentified	Unknown		
56	5764	5934	41.5	6.3	4.45	AGTGAGGGAtgag	Unknown		
						ATG			
68b	5947	6153	30.0	7.7	5.42	AAAGGAGataagaa	S114[a]	57 (39/68)	Y11901
						ATG			
237	6234	6947	38.4	28.1	5.04	AAGGttttag	rlt (ORF19)	73 (155/211)	U38906
						ATG	S114 (ORF1)	30 (64/212)	Y11901
							Tuc2009 (ORF24′)	39 (28/71)	AF109874.1
139a	6944	7363	37.1	15.3	8.78	GGAGGAcggaga	dUTPase		
						ATG	rlt (ORF20)	92 (129/139)	U38906
							S114 (ORF3)	92 (129/139)	Y11901
118a	7366	7722	37.0	13.2	4.47	GGAGGtttgaaa	rlt (ORF21)	50 (29/57)	U38906
						ATG			
65	7709	7906	35.4	7.6	5.74	GATTGGAGGgg	Unknown		
						ATG			
120a	7899	8261	35.8	13.8	4.85	GAAGGGAGcggcg	Unknown		
						ATG			

[a] ORF that was not identified by the authors.

tive methylase gene contained in the genome of phage Tuc2009 (ORF18) is located in a region of 90% homology with ul36 (Fig. 4A) (McGrath *et al.,* 1999) and could have been acquired recently as observed with lactococcal phage ϕ50 (Hill *et al.,* 1991b). It was previously suggested that ϕ50 (P335 species) recombined with a plasmid bearing a restriction-modification system and gained a domain of the methylase that protected it from DNA restriction.

Identification of the phage origins of replication (*ori*)

At the amino acid level, ORF255 of ul36 is highly homologous to the replication protein (ORF16 or Rep2009) of phage Tuc2009 (Table 4). Moreover, a section (coordinates 3608–3768) within the coding region of ORF255 is 100% homologous to the *ori* of Tuc2009. It has been experimentally demonstrated that Rep2009 binds to this *ori* (McGrath *et al.,* 1999). Phage ul36.2 also possesses ORF255 and the putative *ori* of ul36, but in ul36.1, ORF255 is replaced by ORF235 (Fig. 3). Within the coding sequence of ORF235, series of direct repeats, located in an AT-rich region, were identified (Fig. 5). The 1.1-kb *Sca*I-*Dra*I fragment of ul36 containing ORF255 and the 1.1-kb *Pst*I-*Apa*I fragment of ul36.1 carrying ORF235 were cloned in the shuttle vector pMIG3 and the resulting plasmids (pSRQ828 and pSRQ829) were introduced

TABLE 5

General Features of the Putative ORFs of the Sequenced Region of *L. lactis* SMQ-86 and Comparison with ul36 and Databases

ORF	Start	Stop	%G + C	MW (kDa)	pI	SD sequence AGAAAGGAGGA	Function or similarity	Percentage of identity (aa)	Accession no.
111b	496	831	31.5	12.9	7.87	AGAAGGGttcagtaa taaagATG	**ul36 (ORF111a)**	**96 (107/111)**	
							BK5-T (ORF111)	98 (109/111)	L44593
							rlt (ORF6)	95 (104/109)	U38906
78b	1538	1774	36.3	9.1	9.46	AGAAAGGAAAAta ATG	**ul36 (ORF78a)**	**95 (74/78)**	
							phi31 (ORF71)	46 (34/73)	U51128
							Tuc2009 (ORF11)	46 (34/73)	AF109874.1
61b	1793	1978	31.2	7.3	4.94	AAAAAGcacttgcag gcaaATG	**ul36 (ORF61a)**	**92 (56/61)**	
							TP901-1 (ORF9)	94 (54/58)	Y14232
							rlt (ORF8)	89 (52/58)	U38906
							BK5-T (BK5T-Cro)	56 (34/60)	L44593
245	2082	2819	36.2	27.9	4.82	AGAAACGGAGA Atttaaaa ATG	Unknown		
364	2794	3888	33.4	42.3	5.40	Nonidentified	Unknown		
235	4217	4924	30.9	27.1	9.34	GAAATATATAct ATG	Unknown		
73	4905	5126	35.1	8.5	5.30	GGAGGctttaataa ATG	Unknown		
109	5206	5535	37.9	12.5	7.87	Nonidentified	Unknown		
79b	5536	5775	32.1	9.2	9.21	AGGAGGtttga ATG	**ul36 (ORF79a)**	**39 (21/54)**	
							rlt (ORF16)	35 (21/59)	U38906
							Tuc2009 (ORF20)	43 (20/46)	AF109874.1
174	5881	6405	35.8	20.1	9.24	ATAAAGGcaataaa ATG	Unknown		
68c	6419	6625	26.1	7.9	5.26	AAGAAGGAAAAt tagATG	**ul36 (ORF68b)**	**57 (39/68)**	
							S114[a]	100 (68/68)	Y11901
184	6618	7172	36.6	21.2	5.38	GGAGGAcacgaaaa ATG	**ul36 (ORF237)**	**31 (43/138)**	
							S114 (ORF1)	57 (80/138)	Y11901
							rlt (ORF19)	34 (44/126)	U38906
							Tuc2009 (ORF24′)	38 (37/95)	AF109874.1
139b	7169	7588	38.1	15.1	5.15	GGAGGAcggaga ATG	dUTPase **ul36 (ORF139a)**	**94 (130/139)**	
							rlt (ORF20)	97 (136/139)	U38906
							S114 (ORF3)	97 (135/139)	Y11901
118b	7591	1947	37.3	13.4	4.62	GGAGGtgtaaga ATG	**ul36 (ORF118a)**	**79 (84/117)**	
							rlt (ORF21)	84 (21/25)	U38906
64	7944	8138	36.4	7.5	5.74	GGAGGggatag ATG	**ul36 (ORF65)**	**98 (63/64)**	
120b	8131	8493	35.8	13.8	4.85	GAAGGGAGcggcg ATG	**ul36 (ORF120a)**	**100 (120/120)**	

[a] ORF that was not identified by the authors.

into *L. lactis* SMQ-481. It is recognized that the presence of a phage *ori* in *trans* affects the phage development, probably by competing for the replication proteins (Hill *et al.,* 1990; O'Sullivan *et al.,* 1993; McGrath *et al.,* 1999). The

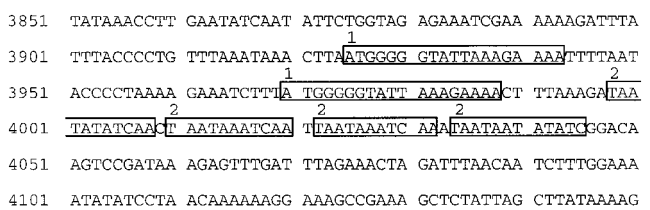

FIG. 5. Origin of replication of phage ul36.1. The G + C content of this region is 25.0%. The direct repeats are in boxes. Matching repeats have the same annotated number.

EOP of several P335-like phages was measured on SMQ-483 (pSRQ828) and SMQ-484 (pSRQ829). The presence of pSRQ828 reduced the EOP and plaque size of ul36, ul36.2, and Q33, while pSRQ829 affected only the EOP and plaque size of ul36.1 (Table 6). The propagation of phages ϕ31, ϕ50, and Q30 was not affected by either of the phage origins, which indicates that these P335-like phages possess functionally different origins of replication.

Hill *et al.* (1990) also demonstrated that a plasmid bearing the phage *ori* replicates at a higher rate during phage infection. To confirm that cloned fragments contained functional origins of replication, total DNA of SMQ-483 and SMQ-484 was isolated during infection by ul36 and ul36.1. The ul36 infection induced replication of

TABLE 6

Effect of Plasmids pSRQ828 and pSRQ829 on the EOP of Different Phages of the Lactococcal P335 Species

Strain	ul36	ul36.1	ul36.2	ϕ31	ϕ50	Q30	Q33
SMQ-483 (pSRQ828)	0.5[a]	1[b]	0.4[a]	1	1	1	0.7[c]
SMQ-484 (pSRQ829)	1[b]	0.5[a]	1[b]	1	1	1	1[d]

[a] Pinpoint plaques.
[b] 0.5 to 1.0-mm plaques.
[c] Pinpoint to 0.5-mm plaques.
[d] 1.0 to 1.5-mm plaques.

pSRQ828 but did not increase the copy number of pSRQ829 (Fig. 6). Inversely, ul36.1 infection only enhanced the replication of pSRQ829. These results are in agreement with the plaquing assays and confirm that ul36.1 possesses a distinct *ori*.

DISCUSSION

This study describes two different genetic exchanges between the lytic phage ul36 and the chromosome of its host strain. The chromosomal fragment has homology with P335-like phages, suggesting that this DNA is part of a prophage. Addition of mitomycin C at various concentrations failed to cause the host lysis (data not shown), indicating that this prophage may be incomplete or noninducible. The boundaries of the substituted DNA in ul36.1 and ul36.2 corresponded to regions of highest homology between ul36 and the chromosome, which supports the fact that the exchanges occurred by homologous recombination. Shen and Huang (1986) have demonstrated that the rate of homologous recombination is correlated to the length and percentage of homology of the DNA sequences involved and that over 23–27 bp of 100% homology are needed for recombination. According to these criteria, other potential junction points within ul36 and SMQ-86 were present, but it is not known whether their use would have led to viable phages.

To our knowledge, this is the first report on the mode of DNA exchange in lactococcal phages. However, it has been demonstrated that some λ mutants had also acquired heterologous DNA from defective prophages by homologous recombination (Gottesman *et al.,* 1974; Gillen *et al.,* 1977; Fisher and Feiss, 1980; Kaiser, 1980). In all cases, the new genes or *cis*-acting sequences had the same overall function in the phage lytic cycle than the original elements. The recombinant mutants were, in fact, revertants of laboratory-engineered λ mutants. By opposition, ul36.1 and ul36.2 are recombinant mutants of a natural phage and have a selective advantage resulting from the acquisition of novel characteristics. Both are insensitive to AbiK and ul36.1 has a reduced burst size and a different origin of replication. Interestingly, the two last features do not relate to the selective pressure used to obtain the mutants. The decreased burst size may be somewhat disadvantageous for the phage, but the new

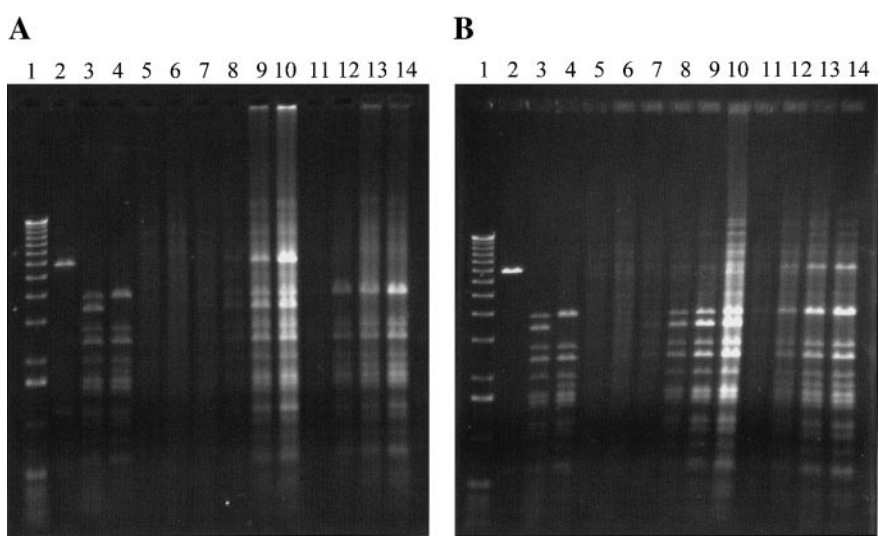

FIG. 6. DNA replication assays in phage-infected *L. lactis* cells containing the origin of replication of phage ul36 (A) and of phage ul36.1 (B). Lane 1, 1-kb ladder (Gibco/BRL); lane 2, plasmid of the strain (pSRQ828 in panel A, pSRQ829 in panel B); lane 3, ul36; lane 4, ul36.1; lanes 5 and 6, DNA of uninfected cells at $t = 0$ and $t = 75$ min; lanes 7 to 10, DNA of cells infected with ul36 at $t = 0$, 45, 60, and 75 min, respectively; lanes 11 to 14, DNA of cells infected with ul36.1 at $t = 0$, 45, 60, and 75 min, respectively. All DNA samples were digested with *Hin*dIII.

origin can confer an advantage toward an *ori*-based antiphage mechanism. It is also conceivable that the *ori* of ul36.1 may be less efficient and thus be responsible for the reduced burst size.

The findings that phages of the P335 species can evolve by homologous recombination events are of particular interest for two reasons. First, the P335 species is the only group among the three common lactococcal species that has temperate and lytic phages. Because phage-related sequences seem to be widespread in *Lactococcus* (Bolotin *et al.,* 1999), this mode of DNA replacement may be prevalent in the P335 species. Exchanges through homologous recombination could also occur between two lytic phages of a species that does not comprise temperate members, but coinfection is thought to be rare in natural environments in comparison to infection of lysogens and defective lysogens (Campbell, 1988).

Second, as opposed to the 936- and c2-like phages, the P335-like phages seem to be more heterogeneous with respect to DNA-DNA hybridization, structural protein profiles, and the presence of two modes of DNA packaging within this species (Jarvis, 1995; Schouler, 1996; Labrie and Moineau, 2000). The gene pool of this phage group appears to be varied enough to allow changes in phenotypes and also to serve as rescue device in case of the inactivation of key genes or proteins. In the sequenced regions of ul36 and the chromosome, the products of 11 out of the 24 different putative genes had no significant homology with the proteins in the databases, including those from several lactococcal phages. Significant DNA homology (>50%) with other P335-like phages was limited to short blocks of genes or even intergenic regions (Fig. 4) and the degree of homology varied greatly according to the phage member. The genetic diversity within this species is also well demonstrated by the origins of replication. The *ori*s of phages ϕ31 (Per31) and ϕ50 (Per50) were previously cloned and conferred resistance to cells against infection by phages ϕ31 and ϕ50, respectively (O'Sullivan *et al.,* 1993). The *ori*s described in this study do not impair the development of ϕ31 and ϕ50. Furthermore, the *ori* of ϕ50 has been sequenced and is not homologous to that of either ul36 or ul36.1. The absence of cross-resistance implies the existence of at least four functionally different origins of replication in the P335 species.

Other mechanisms of evolution are certainly intervening in this phage group as mutants bearing point mutations were also described elsewhere (Dinsmore and Klaenhammer, 1997). Nucleotide substitution represents a particular strategy for the steady development or improvement of a biological function (Arber, 1991). Clearly, this process is frequent but it is far from being as efficient as the acquisition of genetic information containing already functional properties. In fact, gene acquisition is probably the most efficient way to generate unique phages (Arber, 1991). The two large units of genetic exchange described here concur with the modular theory of evolution proposed for the lambdoid phages, where the unit of the evolutionary exchange is a group of functional, but noninterchangeable, genes (Botstein, 1980). However, DNA-DNA comparisons with other P335 phages also revealed that segments or genes of a functional module could be interchangeable. For example, ORF131, ORF252, and ORF141 of ul36 may represent a module, since they were replaced by ORF265 and ORF364 in ul36.2 (Fig. 3). In Tuc2009, ORF141 is present but ORF131 and ORF252 are substituted for ORF13 and ORF14 (Fig. 4A). In TP901–1, homologs of ORF131 (ORF10) and ORF141 (ORF12′) are present but ORF252 is replaced with ORF11, which is a homolog of ORF14 from Tuc2009 (Fig. 4A). Thus, as suggested by Neve *et al.* (1998) for *Streptococcus thermophilus* phages, exchangeable modules could be as short as one gene or even segments of genes.

In summary, contrary to c2- and 936-like phages, genetic exchanges that use homologous sequences are probably more frequent among P335-like phages because of the presence of prophages or remnants of prophages in their hosts. These exchanges have a greater potential to create phages with new phenotypes, considering the genome variability and flexibility within the species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in LB broth and *Lactococcus lactis* strains were grown at 30°C in M17 supplemented with 0.5% glucose (GM17). When needed, antibiotics were added to the media: 50 μg ampicillin and 20 μg chloramphenicol/mL for *E. coli*; 5 μg chloramphenicol and 5 μg erythromycin/mL for *L. lactis*.

Bacteriophage isolation, propagation, and assays

Phages used in this study are listed in Table 1. They were isolated and propagated as described previously (Moineau *et al.,* 1994). The efficiency of plaquing (EOP) was measured according to Sanders and Klaenhammer (1980). One-step growth curves were performed as outlined previously (Moineau *et al.,* 1993). The burst size was determined by dividing the average titer after 105 min by the average titer before 60 min.

DNA isolation and manipulations

Phage DNA was obtained with the Qiagen Lambda Maxi Kit (Qiagen, Chatsworth, CA). *E. coli* plasmid DNA was isolated with Qiagen Plasmid Maxi Kit (Qiagen). *L. lactis* plasmid DNA was isolated by the method of O'Sullivan and Klaenhammer (1993). Total DNA of SMQ-86 was recovered through the use of the same

method but with the following modifications: NaOH was omitted and KoAc 3M was adjusted to pH 7.0. Total DNA of infected cells was obtained as described by Hill *et al.* (1991a). DNA hybridization was conducted as follows: total DNA of *L. lactis* SMQ-86 was digested; restriction fragments were separated by electrophoresis on agarose gel (0.8%) and transferred on a nylon membrane. Probes were labeled with DIG-High prime kit (Roche Diagnostics, Laval, Québec, Canada); prehybridization, hybridization, washes, and detection by chemiluminescence proceeded as suggested by the manufacturer. DNA manipulations and cloning were done as described by Sambrook *et al.* (1989). *E. coli* and *L. lactis* cells were electroporated with the Gene Pulser II (Bio-Rad Laboratories, La Jolla, CA) as described by the manufacturer and Holo and Nes (1989), respectively.

DNA sequencing and analysis

Phage restriction fragments were cloned in pBS and sequenced using universal primers. The sequence was completed by primer walking by the use of the total phage genome as a template. Primers used to sequence the DNA of phage ul36.1 were also used to sequence the PCR products from *L. lactis* SMQ-86. To retrieve the flanking chromosomal regions, inverse PCR was performed following digestion of the total DNA from SMQ-86 with *Pst*I or *Eco*RI, dilution of the restricted DNA, self-ligation, phenol-chloroform extraction, and DNA precipitation. DNA was sequenced on both strands with an Applied Biosystems 373A automated DNA sequencer by the use of synthetic oligonucleotide primers as well as the universal forward and reverse primers (Applied Biosystems 394 DNA/RNA Synthetizer). DNA and deduced protein sequences were analyzed with the Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI) (Devereux *et al.,* 1984). The sequences are available under the Accession numbers AF212844 (SMQ-86), AF212845 (ul36), AF212846 (ul36.1), and AF212847 (ul36.2).

Structural protein profile

The method of Braun *et al.* (1989) was used to determine the phage structural protein profiles. Proteins were separated through a sodium dodecyl sulfate–15% polyacrylamide minigel at 100 V with the Mini-Protean II apparatus (Bio-Rad Laboratories) as outlined previously (Chibani Azaïez *et al.,* 1998).

Electron microscopy

Purified phages were deposited on a carbon-coated Formvar grid and mixed with an equal volume of 2% potassium phosphotungstate (pH 7.2). Phages were observed with a Philips EM 300 electron microscope.

ACKNOWLEDGMENTS

We are grateful to H.-W. Ackermann for the electron microscopy observations. We thank E. Emond for critical reading of the manuscript. J.D.B. is a recipient of a Fonds FCAR graduate scholarship. This work was supported by a strategic grant from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

Alatossava, T., and Klaenhammer, T. R. (1991). Molecular characterization of three small isometric-headed bacteriophages which vary in their sensitivity to the lactococcal phage resistance plasmid pTR2030. *Appl. Environ. Microbiol.* **57**, 1346–1353.

Arber, W. (1991). Elements of microbial evolution. *J. Mol. Evol.* **33,** 4–12.

Bissonnette, F., Labrie, S., Deveau, H., Lamoureux, M., and Moineau, S. (2000). Characterization of mesophilic mixed starter cultures used for the manufacture of aged Cheddar cheese. *J. Dairy Sci.* (in press).

Bolotin, A., Mauger, S., Malarma, K., Ehrlich, S. D., and Sorokin, A. (1999). Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. *Antonie Van Leeuwenhoek* **76,** 27–76.

Botstein, D. (1980). A theory of modular evolution for bacteriophages. *Ann. N.Y. Acad. Sci.* **354,** 484–490.

Braun, V., Hertwig, S., Neve, H., Geis, A., and Teuber, M. (1989). Taxonomic differentiation of bacteriophages of *Lactococcus lactis* by electron microscopy, DNA-DNA hybridization, and protein profiles. *J. Gen. Microbiol.* **135,** 2551–2560.

Campbell, A. (1988). Phage evolution and speciation. *In* "The Bacteriophages" (R. Calendar, Ed.), pp. 1–14. Plenum Press, New York.

Campbell, A. (1994). Comparative molecular biology of lambdoid phages. *Ann. Rev. Microbiol.* **48,** 193–222.

Casjens, S., Hatfull, G., and Hendrix, R. (1992). Evolution of dsDNA tailed-bacteriophage genomes. *Semin. Virol.* **3,** 383–397.

Chibani Azaïez, S. R., Fliss, I., Simard, R. E., and Moineau, S. (1998). Monoclonal antibodies raised against native major capsid proteins of lactococcal c2-like bacteriophages. *Appl. Environ. Microbiol.* **64,** 4255–4259.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12,** 387–395.

Dinsmore, P. K., and Klaenhammer, T. R. (1997). Molecular characterization of a genomic region in a *Lactococcus* bacteriophage that is involved in its sensitivity to the phage defense mechanism AbiA. *J. Bacteriol.* **179,** 2949–2957.

Djordjevic, G. M., and Klaenhammer, T. R. (1997). Genes and gene expression in *Lactococcus* bacteriophages. *Int. Dairy J.* **7,** 489–508.

Émond, E., Holler, B. J., Boucher, I., Vandenbergh, P. A., Vedamuthu, E. R., Kondo, J. K., and Moineau, S. (1997). Phenotypic and genetic characterization of the bacteriophage abortive infection mechanism AbiK from *Lactococcus lactis*. *Appl. Environ. Microbiol.* **63,** 1274–1283.

Fisher, R., and Feiss, M. (1980). Reversion of cohesive end site mutant of bacteriophage λ by recombination with a defective prophage. *Virology* **107,** 160–173.

Gillen, J. R., Karu, A. E., Nagaishi, H., and Clark, A. J. (1977). Characterization of the deoxyribonuclease determined by lambda reverse as exonuclease VIII of *Escherichia coli*. *J. Mol. Biol.* **113,** 27–41.

Gottesman, M. M., Gottesman, M. E., Gottesman, S., and Gellert, M. (1974). Characterization of bacteriophage λ reverse as an *Escherichia coli* phage carrying a unique set of host-derived recombination functions. *J. Mol. Biol.* **88,** 471–487.

Hill, C., Massey, I. J., and Klaenhammer, T. R. (1991a). Rapid method to characterize lactococcal bacteriophage genomes. *Appl. Environ. Microbiol.* **57,** 283–288.

Hill, C., Miller, L. A., and Klaenhammer, T. R. (1990). Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis*. *J. Bacteriol.* **172,** 6419–6426.

Hill, C., Miller, L. A., and Klaenhammer, T. R. (1991b). *In vivo* exchange of a functional domain from a type IIA methylase between lactococcal plasmid pTR2030 and a virulent bacteriophage. *J. Bacteriol.* **173,** 4363–4370.

Holo, H., and Nes, I. F. (1989). High frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* **55,** 3119–3123.

Huang, X., and Miller, W. (1991). A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math.* **12,** 337–357.

Jarvis, A. W. (1995). Relationships by DNA homology between lactococcal phages 7–9, P335 and New Zealand industrial lactococcal phages. *Int. Dairy J.* **5,** 355–366.

Jarvis, A. W., Fitzgerald, G. F., Mata, M., Mercenier, A., Neve, H., Powell, I. A., Ronda, C., Saxelin, M., and Teuber, M. (1991). Species and type phages of lactococcal bacteriophages. *Intervirology* **32,** 2–9.

Kaiser, K. (1980). The origin of Q-independent derivatives of phage λ. *Mol. Gen. Genet.* **179,** 547–554.

Labrie, S., and Moineau, S. (2000). Multiplex PCR for the detection, and the identification of lactococcal bacteriophages. *Appl. Environ. Microbiol*. (in press).

Madsen, P. L., and Hammer, K. (1998). Temporal transcription of the lactococcal temperate phage TP901–1 and DNA sequence of the early promoter region. *Microbiology* **144,** 2203–2215.

McGrath, S., Seegers, J. F. M. L., Fitzgerald, G. F., and van Sinderen, D. (1999). Molecular characterization of a phage-encoded resistance system in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **65,** 1891–1899.

Moineau, S. (1999). Applications of phage resistance in lactic acid bacteria. *Antonie Van Leeuwenhoek* **76,** 377–382.

Moineau, S., Borkaev, M., Holler, B. J., Walker, S. A., Kondo, J. K., Vedamuthu, E. R., and Vandenbergh, P. A. (1996). Isolation and characterization of lactococcal bacteriophages from U.S. buttermilk plants. *J. Dairy Sci.* **79,** 2104–2111.

Moineau, S., Durmaz, E., Pandian, S., and Klaenhammer, T. R. (1993). Differentiation of two abortive infection mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Appl. Environ. Microbiol.* **59,** 208–212.

Moineau, S., Fortier, J., Ackermann, H.-W., and Pandian, S. (1992). Characterization of lactococcal bacteriophages from Québec cheese plants. *Can. J. Microbiol.* **38,** 875–882.

Moineau, S., Pandian, S., and Klaenhammer, T. R. (1994). Evolution of a lytic bacteriophage via DNA acquisition from the *Lactococcus lactis* chromosome. *Appl. Environ. Microbiol.* **60,** 1832–1841.

Neve, H., Zenz, K. I., Desiere, F., Koch. A., Heller, K. J., Brussow, H. (1998). Comparison of the lysogeny modules from the temperate *Streptococcus thermophilus* bacteriophages TP-J34 and Sfi21: Implications for the modular theory of phage evolution. *Virology* **241,** 61–72.

O'Sullivan, D. J., Hill, C., and Klaenhammer, T. R. (1993). Effect of increasing the copy number of bacteriophage origins of replication, in *trans*, on incoming-phage proliferation. *Appl. Environ. Microbiol.* **59,** 2449–2456.

O'Sullivan, D. J., and Klaenhammer, T. R. (1993). Rapid mini-prep isolation of high quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl. Environ. Microbiol.* **59,** 2730–2733.

Prévots, F., Tolou, S., Delpech, B., Kaghad, M., and Daloyau, M. (1998). Nucleotide sequence and analysis of the new chromosomal abortive gene *abiN* of *Lactococcus lactis* subsp. *cremoris* S114. *FEMS Microbiol. Lett.* **159,** 331–336.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanders, M. E., and Klaenhammer, T. R. (1980). Restriction/modification in group N streptococci: Effect of heat on development of modified lytic bacteriophage. *Appl. Environ. Microbiol.* **40,** 500–506.

Schouler, C. (1996). Genomic organization of lactic acid bacteria phages. *Lait* **76,** 81–89.

Shen, P., and Huang, H. V. (1986). Homologous recombination in *Escherichia coli*: Dependence on substrate length and homology. *Genetics* **112,** 441–457.

van Sinderen, D., Karsens, H., Kok, J., Terpstra, P., Ruiters, M. H. J., Venema, G., and Nauta, A. (1996). Sequence analysis and molecular characterization of the temperate lactococcal bacteriophage r1t. *Mol. Microbiol.* **19,** 1343–1355.

Wells, J. M., Wilson, P. W., and LePage, R. W. F. (1993). Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J. Appl. Bacteriol.* **74,** 629–636.